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Production of Hepatitis C Virus Lacking the Envelope-Encoding Genes for Single-Cycle Infection by Providing Homologous Envelope Proteins or Vesicular Stomatitis Virus Glycoproteins in *trans*▽†

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Hepatitis C virus (HCV) infection is a major worldwide health problem. The envelope glycoproteins are the major components of viral particles. Here we developed a *trans*-complementation system that allows the production of infectious HCV particles in whose genome the regions encoding envelope proteins are deleted (HCVΔE). The lack of envelope proteins could be efficiently complemented by the expression of homologous envelope proteins in *trans*. HCVΔE production could be enhanced significantly by previously described adaptive mutations in NS3 and NS5A. Moreover, HCVΔE could be propagated and passaged in packaging cells stably expressing HCV envelope proteins, resulting in only single-round infection in wild-type cells. Interestingly, we found that vesicular stomatitis virus (VSV) glycoproteins could efficiently rescue the production of HCV lacking endogenous envelope proteins, which no longer required apolipoprotein E for virus production. VSV glycoprotein-mediated viral entry could allow for the bypass of the natural HCV entry process and the delivery of HCV replicon RNA into HCV receptor-deficient cells. Our development provides a new tool for the production of single-cycle infectious HCV particles, which should be useful for studying individual steps of the HCV life cycle and may also provide a new strategy for HCV vaccine development.

Hepatitis C virus (HCV) is a major etiological agent of severe liver diseases, including liver cirrhosis and hepatocellular carcinoma, with an estimated 170 million people infected worldwide (2). No vaccine is available to prevent HCV infection, and the sole therapeutic treatment available, based on interferon, does not always lead to cure and is often associated with significant side effects (12). HCV is an enveloped plus-strand RNA virus belonging to the family *Flaviviridae*. The 9.6-kb viral genome encodes a single polyprotein that is co- or posttranslationally cleaved into structural (core, E1, and E2) and nonstructural (p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) proteins (4). The structural proteins encapsidate the viral genome into infectious particles and mediate the entry of the virus into permissive cells; the nonstructural proteins NS3, NS4A, NS4B, NS5A, and NS5B are the viral components of the membrane-bound replication complexes that catalyze genomic RNA replication (18).

HCV envelope glycoproteins E1 and E2 are processed by cellular signal peptidases in the endoplasmic reticulum (ER), are highly glycosylated in the amino-terminal ectodomains, and are anchored to the membrane by the carboxyl-terminal transmembrane domains to form a stable noncovalent heterodimer complex. This oligomer is thought to be the prebudding form of the functional HCV glycoproteins and is essential for interaction with the receptor during HCV entry (52). According to the current model for HCV assembly and secretion, HCV core particles containing the genome assemble in the lipid droplets and acquire the viral envelope by budding into the ER (43), during which time the E1 and E2 proteins are inserted into the viral envelope (15, 45).

The development of HCV infection models that reproduce the entire HCV life cycle *in vitro* has created an opportunity to study each viral protein as a determinant of virus production (31, 53, 57). A number of recent studies have demonstrated that besides the structural proteins core, E1, and E2, the nonstructural proteins NS3 and NS5A, as well as p7 and NS2, also play important roles in virus assembly and secretion (3, 25, 32, 36, 37, 41, 48). Moreover, accumulating evidence suggests that the association between HCV and host low-density lipoprotein (LDL) or very low density lipoprotein (VLDL) is important for virus egress and that apolipoprotein E (apoE), a component of LDL/VLDL, is required for HCV infectivity and production (5, 9, 10, 23, 49). These results indicate that HCV assembly and release are mediated by a concerted interplay between viral structural proteins, nonstructural proteins, and host factors.

trans-complementation systems have been utilized as a reverse-genetics approach for studying the roles of individual HCV proteins in the viral life cycle independently of their *cis*-acting effects. For instance, HCV core protein with lethal mutations could be rescued by ectopic expression of wild-type or C-terminally truncated core proteins (28, 36). HCV genomes with a deletion in p7 could be rescued by the expression of p7 either with or without the leading signal sequence (8). Mutations in NS2 blocking virus assembly could be rescued by expression of NS2 in *trans* from a helper replicon (24, 56). Mutations in NS5A domain III disrupting virus production

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could be rescued by a helper replicon expressing functional NS5A (3). HCV subgenomic replicon RNA lacking the entire region encoding the structural proteins could produce infectious viruses upon expression of the structural proteins in *trans* from helper viruses, stably expressing cell lines, or transient plasmid transfection (1, 22, 33, 40, 47). However, complementation of HCV with a deletion of the envelope gene by envelope glycoproteins provided alone in *trans* has not been reported yet.

Here we developed a *trans*-complementation system based on HCV envelope glycoproteins that allows the production of single-round infectious HCV particles (HCVΔE). The lack of the envelope could be complemented by the expression of envelope proteins in *trans* from transient plasmid transfection or in packaging cells stably expressing HCV envelope proteins alone. HCVΔE could be propagated and passaged in the packaging cells while resulting in only single-round infection in wild-type cells. In addition, we observed that vesicular stomatitis virus glycoproteins (VSV-G) could rescue the production of HCV lacking endogenous envelope proteins. Further characterization of these pseudotype viruses (HCVvsv) revealed that HCVvsv entry was indeed mediated by VSV glycoproteins and that HCVvsv secretion did not require apoE. The wide host range of VSV glycoprotein-mediated infection would allow for the bypass of the natural HCV entry process and the delivery of HCV replicon RNA into HCV receptor-deficient cells. Taken together, our development provided a new tool for producing single-cycle infectious HCV particles, which should be useful in the study of particular steps of the HCV life cycle. This technology may also provide a new strategy for the establishment of HCV replicon cell lines and vaccine development.

MATERIALS AND METHODS

Cells and viruses. The hepatic cell lines (Huh7 and Huh7.5.1) were maintained in complete Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum, 10 mM HEPES buffer, 100 U/ml penicillin, and 100 mg/ml streptomycin. Huh7.5.1E packaging cells were produced by transfecting 2 μg of the pcDNA3-JFH1-E1/E2 plasmid into 8×10^5 Huh7.5.1 cells, followed by 3 weeks of selection with 500 μg/ml G418. The cell clone with the highest E2 protein expression levels was expanded and used for the studies. To generate an apoE knockdown cell line, a lentiviral vector encoding short hairpin RNA (shRNA) targeting apoE (5′-AGACAGAGCCGGAGCCCGA-3′) was cotransfected with plasmids encoding compatible packaging proteins and VSV-G into HEK293 cells, as described previously (14). At 72 h posttransfection, cell supernatants were collected, filtrated, and used to transduce Huh7.5.1 cells. A control cell line expressing shRNA targeting firefly luciferase (5′-CGTACGCGGAATACTTCGA-3′) was generated in the same way.

Plasmids. Plasmids pUC-JFH1-delE and pFGR-JFH1-delE, which contain an in-frame deletion in the regions of JFH1 encoding E1 and E2, have been described previously (11, 53). Plasmid pcDNA3-JFH1-E1/E2, expressing JFH1 envelope glycoproteins, was constructed by PCR amplification of the JFH1 E1 and E2 regions (amino acid residues 171 to 750) and insertion of the PCR product into pcDNA3.1. The plasmids encoding the envelope proteins of other HCV strains were generated similarly. Plasmid pLP/VSV-G, expressing the glycoproteins of vesicular stomatitis virus, was obtained from Invitrogen (Carlsbad, CA). All plasmids constructed were verified by DNA sequencing.

Indirect immunofluorescence. Intracellular immunostaining was performed as described previously (57). Briefly, the cells were fixed with 4% paraformaldehyde and were permeabilized with 0.5% Triton X-100. HCV E2, core, NS5A, and VSV-G were stained by using a human monoclonal anti-E2 antibody (C1) (17), a mouse monoclonal anti-core antibody (C7-50; Abcam, Cambridge, United Kingdom), a rabbit polyclonal anti-NS5A antibody (a generous gift from Kunitada Shimotohno, Kyoto University, Kyoto, Japan), and a monoclonal anti-VSV-G antibody (P5D4; Abcam), respectively. Bound primary antibodies were detected by using Alexa Fluor 488- or Alexa Fluor 555-conjugated secondary antibodies (Molecular Probes, Eugene, OR). Nuclei were stained with Hoechst dye.

Western blot analysis. Cells were collected in radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 50 mM Tris [pH 8], 1% NP-40, 0.5% deoxycholate, and 1% sodium dodecyl sulfate [SDS]) and were quantified by a bicinchoninic acid (BCA) assay (Pierce, Rockford, IL). Cell lysate proteins were separated by 12% SDS-polyacrylamide gel electrophoresis (PAGE) and were then transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA). Membranes were probed first with a primary antibody against HCV E1 (made in-house), E2 (Biodesign International, Saco, ME), NS3 (8G-2; Abcam), or β-actin (Sigma, St. Louis, MO) and then with alkaline phosphatase-conjugated goat anti-rabbit, donkey anti-goat, or goat anti-mouse secondary antibodies (Promega, Madison, WI). Proteins were visualized by a 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (BCIP)–nitroblue tetrazolium kit (Promega).

HCV infectivity titer and RNA quantification. HCV infectivity titers were determined with Huh7.5.1 cells by endpoint dilution and immunostaining as described previously (57). HCV RNA levels were determined by quantitative reverse transcription-PCR (RT-PCR) as described previously (17).

Density gradient ultracentrifugation. Gradients were formed by overlaying 2 ml of 20%, 30%, 40%, 50%, and 60% sucrose solutions in TNE buffer (10 mM Tris-HCl [pH 8], 150 mM NaCl, 2 mM EDTA) as described previously (17). Equilibrium was reached by ultracentrifugation for 16 h at 30,000 rpm ($154{,}000 \times g$) in an SW41 Ti rotor at 4°C in a Beckman Optima L-80XP preparative ultracentrifuge. Fifteen gradient fractions of 750 μl were collected from the top, and their infectivity titers and HCV RNA levels were determined as described above. The density of each fraction was determined by measuring the mass of 100-μl aliquots of each sample.

HCV infection kinetics assay. Eighty thousand Huh7.5.1 or Huh7.5.1E cells were seeded into 12-well plates, left overnight, and then inoculated with the virus at a multiplicity of infection (MOI) of 0.01. The infected cells reached confluence on day 4 postinfection and were then split at a ratio of 1:3 into 12-well plates (harvested on day 6), 6-well plates (harvested on day 8), and T25 flasks (harvested on day 10). Culture supernatants were collected at the time points indicated in the figures, and infectivity titers were determined as described above.

Transwell-based *trans*-complementation assay. Eighty thousand Huh7.5.1E cells were seeded in 12-well plates, left overnight, and then inoculated with HCVΔE at an MOI of 0.05. On day 4 postinfection, the infected cells were collected and reseeded at $2 \times 10^4/\text{cm}^2$ on a permeable membrane (pore size, 1.0 μm) in the upper chamber of a transwell system (BD Falcon; Bedford, MA), and naïve Huh7.5.1 cells were seeded at $2 \times 10^4/\text{cm}^2$ in the lower chamber of the transwell system. After the percentage of infected Huh7.5.1 cells reached more than 50% at day 6 postcoculture, the infected Huh7.5.1 cells were collected and reseeded at 2×10^5/well in 24-well plates for the transfection of HCV or VSV glycoproteins using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. The culture supernatants were collected at day 3 posttransfection, and viral infectivity was determined.

Preparation of anti-VSV-G serum. We used virus-like particles (VLP) expressing VSV-G as an immunogen to generate anti-VSV-G serum. To produce VLP, 4.5×10^6 HEK293T cells were cotransfected with 14 μg of pCMVΔR8.2 (38) and 10 μg of the VSV-G plasmid using a calcium phosphate precipitation method. The VLP-containing supernatants were harvested at 16 h posttransfection, loaded onto a 20% sucrose cushion, and ultracentrifuged at 20,000 rpm for 2.5 h at 4°C in a Beckman SW28 rotor. The pellets were resuspended in phosphate-buffered saline (PBS) and were stored at −80°C until use. To generate anti-VSV-G immune sera, female BALB/c mice were injected intraperitoneally with total 200 μl VLP expressing VSV-G in both the prime and boost injections (separated by a 3-week interval). Seven days after the boost, serum samples were collected, heat inactivated at 56°C, and stored in aliquots at −80°C.

Blockade of HCV infection. The blockade of HCV infection by a human monoclonal anti-E2 antibody (C1) (17) or a mouse anti-VSV-G serum was performed as described previously (49). The infection efficiency was determined 3 days postinfection by counting the number of NS5A-positive foci (cell culture-grown HCV [HCVcc]) or cells (HCVΔE and HCVvsv).

Preparation of MLVpp. Human immunodeficiency virus (HIV)-based murine leukemia virus pseudoparticles (MLVpp) were generated as described previously (54). Briefly, 293T cells were cotransfected with plasmids encoding HIV packaging proteins, an HIV vector containing luciferase, and MLV glycoproteins. Supernatants were harvested at 72 h posttransfection and were filtered. Infection was quantified by measuring luciferase activity on a GloMax 96 microplate luminometer (Promega).

HCV secretion assay. About 1×10^5 apoE knockdown cells plated in 24-well plates were infected with the HCVΔE virus at an MOI of 0.5. The cells were washed with warm medium to remove the initial virus inocula on the following

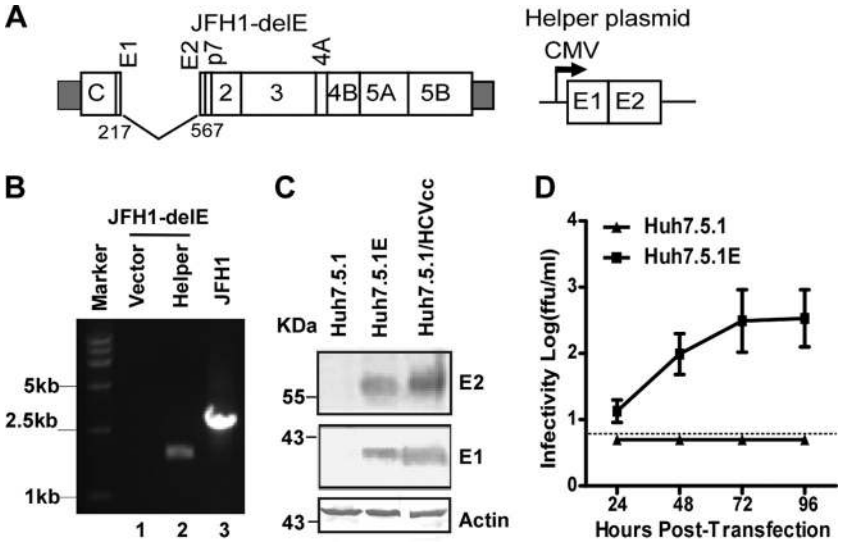

FIG. 1. JFH1 with a deletion in the envelope genes could be rescued by expression of envelope proteins in *trans*. (A) Schematic representations of the JFH1-delE RNA genome, carrying an in-frame deletion of 350 amino acids within the regions encoding E1 and E2, and a helper plasmid expressing the full-length E1 and E2 proteins. The amino acid positions of deletion sites within the envelope region are indicated as 217 to 567. (B) RT-PCR analysis of the HCV genome in the supernatants of Huh7.5.1 cells transfected with JFH1-delE RNA and an empty vector (lane 1), JFH1-delE RNA and the helper plasmid (lane 2), or wild-type JFH1 RNA (lane 3) using a primer set flanking the envelope-encoding region. The DNA size marker is shown on the right. (C) Detection of E1 and E2 expression in Huh7.5.1E packaging cells by Western blot analysis. Proteins were separated from Huh7.5.1E cell lysates by 12% SDS-PAGE and were probed with antibodies specific to HCV E1 and E2. The expression of β-actin was examined as a protein loading control. (D) Kinetics of viral infectivity in the supernatants of naïve Huh7.5.1 and Huh7.5.1E cells after transfection of JFH1-delE RNA. Broken lines indicate the detection limit of the titration assay. Means and standard deviations from three independent experiments are shown.

day. Then the cells were transfected with the HCV or VSV envelope glycoproteins using Lipofectamine 2000 (Invitrogen). The culture supernatants were collected at day 3 posttransfection, and viral infectivity and HCV RNA levels were determined.

VSV glycoprotein-mediated HCV replicon colony formation assay. HCV replicon cells stably expressing the Neo-labeled JFH1 genome lacking the regions encoding E1 and E2 were produced by transfecting *in vitro* transcripts of pFGR-JFH1-delE into Huh7 cells as described previously (11). The VSV-G expression plasmids or the empty vector was transfected into the established replicon cells using Lipofectamine 2000 (Invitrogen). At day 3 posttransfection, culture supernatants were used to inoculate 1×10^5 naïve Huh7 or CD81-deficient Huh7 (designated R3 [58]) cells seeded in a 12-well plate. One day postinfection, the culture medium was replaced with complete DMEM containing G418 (800 μg/ml), which was refreshed every 3 days thereafter. Three weeks after transfection, G418-resistant colonies were stained with crystal violet as described previously (58).

RESULTS

Virus production by JFH1 with the envelope gene deleted could be rescued by the expression of envelope proteins in *trans*. Previous studies have shown that a defect in HCV proteins such as core, p7, NS2, and NS5A could be complemented by expressing the missing proteins in *trans* (3, 8, 24, 25, 28, 36, 56). To test whether the function of HCV envelope glycoproteins E1 and E2 could be complemented in *trans*, we constructed JFH1-delE, in which the regions encoding E1 and E2 (from amino acid position 217 to 567) were deleted, and a helper plasmid expressing the full-length JFH1 E1 and E2 proteins under the control of the cytomegalovirus (CMV) promoter (Fig. 1A). These two cassettes included all HCV nonstructural proteins and *cis* elements required for HCV RNA replication and all structural proteins required for viral genome packaging. Therefore, if these two cassettes are coexpressed within the same cell, viral particles containing a defective HCV genome lacking the envelope genes should be produced and should be infectious. We cotransfected the JFH1-delE RNA transcripts with the helper plasmid into Huh7.5.1 cells. As shown in Fig. S1A in the supplemental material, both core and E2 proteins could be detected simultaneously in a small percentage of transfected cells on day 3 posttransfection. Furthermore, the culture supernatants collected from the cotransfected cells contained infectious viruses (~80 infectious units/ml; designated HCVΔE), while the culture supernatants collected from cells cotransfected with JFH1-delE RNA and the empty vector or a plasmid expressing JFH1 E1 or E2 alone possessed no infectivity (see Fig. S1B in the supplemental material). Importantly, Huh7.5.1 cells infected with HCVΔE expressed HCV core proteins but not E2 proteins (data not shown), indicating that the HCV RNA genomes packaged in HCVΔE indeed lacked the envelope-encoding regions. Furthermore, RT-PCR analysis using a primer set outside the envelope-encoding region confirmed that the envelope-encoding region was indeed deleted in the HCVΔE genome, as expected (Fig. 1B).

The efficiency of HCVΔE production from the cotransfection of JFH1-delE RNA with the envelope-expressing helper plasmid was very low (~80 infectious units/ml), likely due to the low percentage of expression of JFH1-delE RNA and envelope proteins within the same cell. To improve the efficiency of HCVΔE production, we transfected the envelope-expressing plasmid into Huh7.5.1cells and selected a G418-resistant cell line that stably expressed JFH1 E1 and E2 proteins. We designated this cell line Huh7.5.1E. Both West-

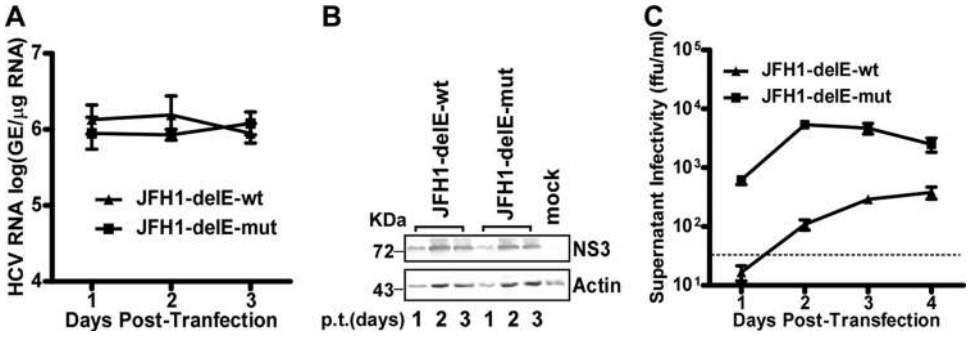

FIG. 2. Adaptive mutations enhanced HCVΔE production. Huh7.5.1E cells were transfected either with wild-type (wt) JFH1-delE or with the JFH1-delE mutant (mut) containing M1051T in NS3 and C2219R in NS5A. (A) Analysis of the replication kinetics of the indicated HCV genomes by quantitative RT-PCR. Means and standard deviations for three independent experiments are shown. (B) Cell lysate proteins were analyzed by Western blot analysis using anti-NS3 and anti-actin antibodies on days 1, 2, and 3 posttransfection (p.t.). (C) The supernatants were collected at days 1, 2, 3, and 4 posttransfection, and their viral infectivity was determined by the titration assay. Broken lines mark the detection limit of the titration assay. Means and standard deviations for three independent experiments are shown.

ern blot and immunofluorescence analyses confirmed the expression of envelope proteins in Huh7.5.1E cells (Fig. 1C; see also Fig. S2 in the supplemental material). Next, we transfected JFH1-delE RNA into Huh7.5.1E cells. The frequencies of coexpression of JFH1-delE RNA and envelope proteins were about 80% (see Fig. S2 in the supplemental material). Importantly, about 300 infectious units/ml of HCVΔE was detected at 72 h posttransfection, while no virus was produced when JFH1-delE RNA was transfected into the parental Huh7.5.1 cells (Fig. 1D).

Taken together, our data showed that the deletion of the envelope genes of JFH-1 could be rescued by the expression of the envelope E1 and E2 proteins in *trans* from transient plasmid transfection or in packaging cells stably expressing these envelope proteins.

Adaptive mutations enhanced HCVΔE production. It has been shown that cell culture-adaptive mutations, especially those in nonstructural proteins, could enhance HCVcc production (27, 55). To further improve the efficiency of HCVΔE production, we engineered two previously described cell culture-adaptive mutations, M1051T in NS3 and C2219R in NS5A, into JFH1-delE. Both mutations were selected from a cell culture with a persistent long-term infection (58) and have been shown to enhance HCVcc production (Y. He and J. Zhong, unpublished data). The wild-type and double mutant JFH1-delE RNAs were electroporated into Huh7.5.1E cells. As shown in Fig. 2A and B, the HCV RNA and NS3 protein levels from day 1 to day 3 posttransfection were similar for wild-type and double mutant JFH1-delE, indicating comparable transfection and HCV genome replication efficiencies. However, as shown in Fig. 2C, the introduction of two mutations into the JFH1-delE genome dramatically improved HCVΔE production. The infectious viruses could be detected as early as 24 h posttransfection and reached a peak titer of 5×10^3 infectious units/ml at 48 h posttransfection. Thus, HCVΔE produced from JFH1-delE with these two mutations was used in subsequent studies.

HCVΔE could propagate in the packaging cells but resulted in only single-cycle infection in naïve cells. Next, we tested whether HCVΔE produced from the RNA transfection experiment could propagate in Huh7.5.1E packaging cells. For this purpose, we infected Huh7.5.1E and Huh7.5.1 cells at an MOI of 0.01 with HCVΔE viral supernatants collected from the transfection experiment for which results are shown in Fig. 2, and we monitored virus production at the time points postinfection indicated in Fig. 3. As shown in Fig. 3A, HCVΔE resulted in productive infection in Huh7.5.1E cells, with amplification kinetics very similar to those of HCVcc with the same adaptive mutations in Huh7.5.1E cells, but produced no infectious viruses in Huh7.5.1 cells. Furthermore, we showed that HCVΔE could be passaged in Huh7.5.1E cells multiple times without losing infectivity (data not shown).

To further confirm the single-cycle-infection nature of HCVΔE, we tested the abilities of HCVΔE and HCVcc to form foci of infection in Huh7.5.1 or Huh7.5.1E cells, since the formation of foci of infected cells is an important marker for productive HCV infection, as previously reported (57). HCVΔE and HCVcc were serially diluted and inoculated into either Huh7.5.1 or Huh7.5.1E cells. The cells were fixed 3 days later and were assayed for HCV core protein expression by immunofluorescence. As shown in Fig. 3B, HCVcc infection resulted in the formation of foci of infected cells (>20 core-positive cells per focus) in both Huh7.5.1 and Huh7.5.1E cells, as expected, whereas HCVΔE infection resulted in focus formation only in Huh7.5.1E cells, not in Huh7.5.1 cells (<6 infected cells per infection origin, likely due to cell division during 3 days of infection), indicating that HCVΔE was unable to produce infectious progeny viruses in Huh7.5.1 cells to infect adjacent cells.

Taken together, these results demonstrated that HCVΔE could propagate and be passaged in the packaging cells but produced only single-cycle infection if no HCV envelope protein was provided in *trans*.

Rescue of HCVΔE production with heterologous HCV envelope proteins or vesicular stomatitis virus glycoproteins. Next, we tested whether HCVΔE production could be rescued by envelope proteins of different HCV strains or a different virus. For this purpose, we used a transwell-based infection system to deliver the JFH1-delE genome into naïve Huh7.5.1 cells. As shown in Fig. 4A, naïve Huh7.5.1 cells seeded in the lower chamber of a transwell system (34) were cocultured with HCVΔE-infected Huh7.5.1E cells grown in the upper chamber

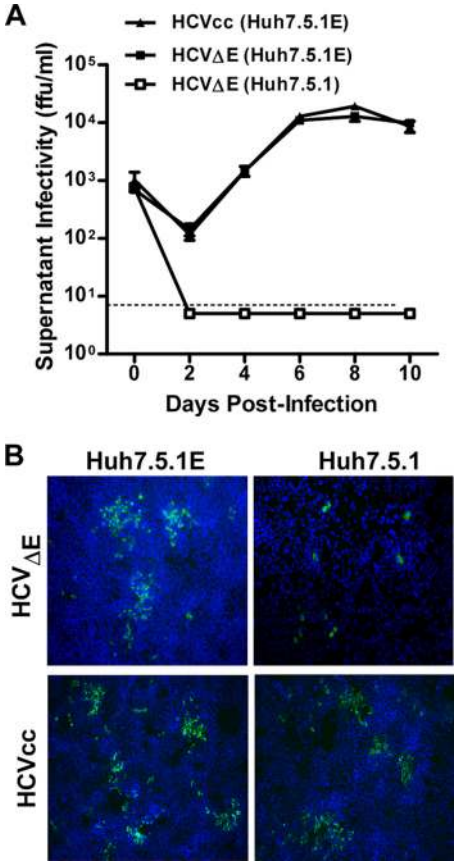

FIG. 3. Single-cycle infection of HCVΔE in naïve Huh7.5.1 cells. (A) Infectious kinetics of HCVΔE and HCVcc in Huh7.5.1 and Huh7.5.1E cells. The cells were inoculated with HCVcc or HCVΔE at an MOI of 0.01. Culture supernatants were harvested at the indicated time points postinfection, and their infectivity was determined by the titration assay. Broken lines mark the detection limit of the titration assay. Means and standard deviations for two independent experiments are shown. (B) Immunofluorescence analysis of core proteins (green) in Huh7.5.1 and Huh7.5.1E cells infected with 100 infectious units of HCVcc or HCVΔE. Nuclei (blue) were stained with Hoechst dye.

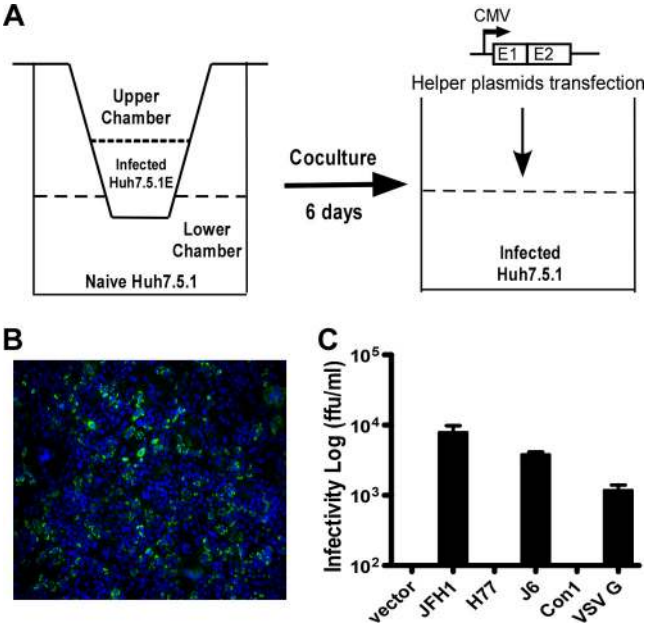

FIG. 4. Production of HCVΔE containing envelope proteins of different HCV strains or VSV. (A) Schematic drawing of the transwell system used for coculturing the HCVΔE-infected packaging cell line (upper chamber) with the naïve Huh7.5.1 cell line (lower chamber). Six days after coculture, the upper chamber was removed, and the cells in the lower chamber were transfected with plasmids expressing envelope glycoproteins. (B) The percentage of HCVΔE-infected Huh7.5.1 cells in the lower chamber was determined by core immunofluorescence analysis at day 6 after coculture. (C) Rescue of HCVΔE by transfection of helper plasmids expressing glycoproteins of different HCV strains or VSV. The culture supernatants were collected at 72 h posttransfection, and the viral infectivity of the supernatants was determined by the titration assay. Means and standard deviations for three independent experiments are shown.

for 6 days until more than 50% of naïve Huh7.5.1 cells had been infected with HCVΔE, as demonstrated by HCV core protein immunostaining (Fig. 4B). Then we withdrew the upper chamber and transfected the HCVΔE-infected Huh7.5.1 cells in the lower chamber with plasmids expressing the envelope proteins of different HCV strains or foreign glycoproteins from VSV. Immunofluorescence analysis of HCV E2 and VSV-G expression on day 3 posttransfection indicated comparable transfection efficiencies (see Fig. S3 in the supplemental material). Culture supernatants were collected on day 3 posttransfection and were analyzed for infectivity by the titration assay. As shown in Fig. 4C, JFH1 and J6 (both genotype 2a strains) envelope proteins could rescue HCVΔE production (8,000 and 4,000 infectious units/ml, respectively), whereas H77 (genotype 1a) and Con1 (genotype 1b) envelope proteins could not. To our surprise, VSV-G was able to rescue HCVΔE production, reaching an infectivity titer of 1,000 infectious units/ml (these pseudotype viruses are designated HCVvsv). These data suggested that chimeric HCVΔE *trans*-packaging could be achieved by providing intragenotypic envelope proteins or foreign envelope proteins (VSV-G) in *trans*.

HCVvsv entry did not depend on interaction between E2 and CD81. Next, we determined whether the entry of HCVΔE and HCVvsv was dependent on the molecular interactions between E2 and CD81. First, we preincubated HCVcc, HCVΔE, and HCVvsv with a human monoclonal anti-E2 antibody (30) or a mouse anti-VSV-G serum for 1 h prior to infection. As shown in Fig. 5A, the anti-E2 antibody inhibited infection by HCVcc and HCVΔE but had no effect on infection by HCVvsv. In contrast, the anti-VSV-G serum efficiently inhibited infection by HCVvsv but had no effect on infection by HCVcc or HCVΔE (Fig. 5B), clearly demonstrating that the entry of HCVΔE and HCVvsv was mediated by HCV and VSV glycoproteins, respectively.

Second, we examined the dependency of HCVΔE and HCVvsv entry on CD81, a critical receptor for HCV. Equal amounts of HCVcc, HCVΔE, and HCVvsv were used to inoculate Huh7 cells and R3 cells, Huh7 derivatives that lack CD81 expression (58). As shown in Fig. 5C, Huh7 cells were equally susceptible to infection by the three viruses. However, R3 cells could be infected only by HCVvsv, not by HCVcc or HCVΔE. These data indicated that HCVvsv infection was no longer restricted by the normal HCV infection tropism. It has been

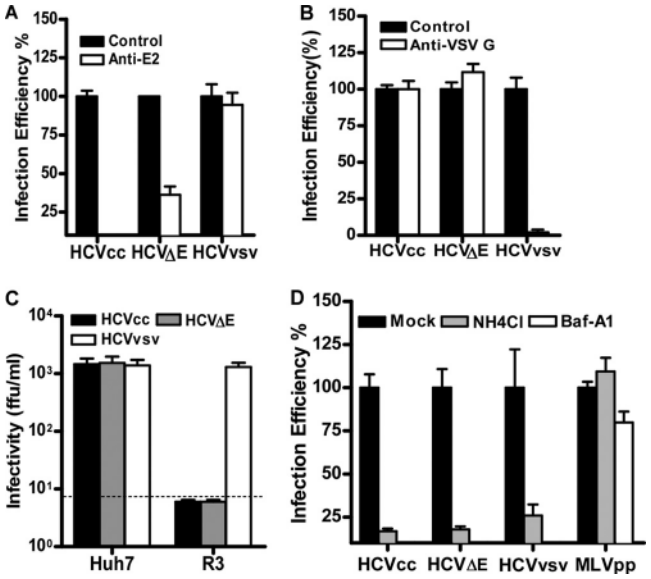

FIG. 5. Characterization of the entry processes of HCVcc, HCVΔE, and HCVvsv. (A and B) Blockade of infection with different HCV particles by an anti-E2 (A) or anti-VSV-G (B) serum. Fifty infectious units of each virus indicated was incubated with a human monoclonal anti-E2 antibody or a mouse anti-VSV-G serum for 1 h prior to inoculation. The infection was analyzed by NS5A immunofluorescence analysis 3 days later, and the number of positive foci (HCVcc) or positive cells (HCVΔE and HCVvsv) was expressed as a percentage of that for the mock treatment control. Error bars represent the standard deviations from three independent experiments. (C) The same amounts of HCV particles were serially diluted and were then inoculated into Huh7 and R3 (CD81$^-$) cells. The infection was analyzed by NS5A immunofluorescence analysis 3 days later. Broken lines mark the detection limit. Means and standard deviations for three independent experiments are shown. (D) Huh7.5.1 cells were either mock treated (filled bars) or incubated for 1 h with a medium containing 10 mM NH_4Cl or 20 nM bafilomycin A1 (Baf-A1) (shaded or open bars, respectively). Then the cells were washed with the medium and were infected with different viruses for 4 h in the presence or absence of the drug. As a control for pH-independent virus entry, infections with lentiviral pseudoparticles bearing MLV envelope proteins were performed in the same way. The infection was analyzed 3 days later by NS5A immunofluorescence analysis except for MLVpp, for which infection efficiency was determined by measuring luciferase activity. Means and standard deviations for three independent experiments are shown.

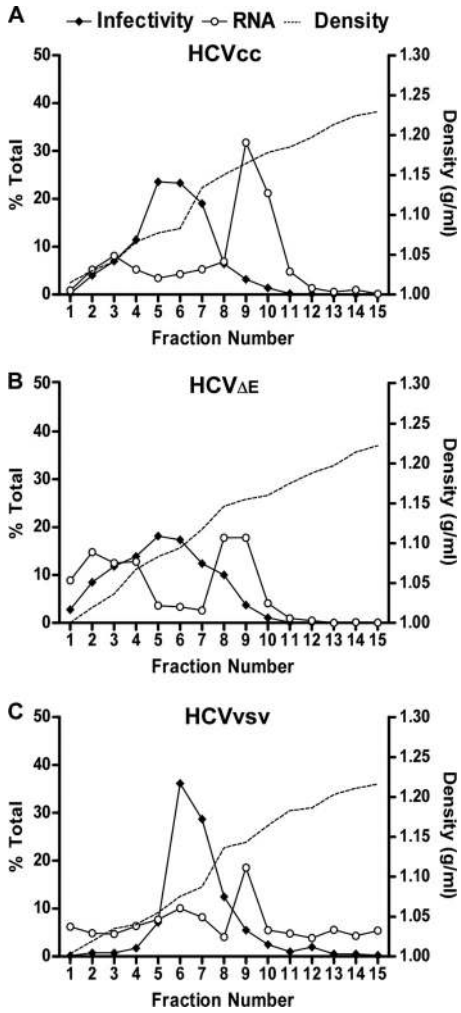

FIG. 6. Characterization of the buoyant densities of HCVcc, HCVΔE, and HCVvsv. The different HCV particles were subjected to a 20% to 60% sucrose gradient. Fifteen fractions were collected from the top, and the infectivity titer and HCV RNA level of each fraction were determined by the titration assay and quantitative RT-PCR. The results are expressed as percentages of the totals for all viruses. The density of each fraction was determined by measuring the mass of a 100-μl aliquot of the fraction. The data shown are representative of results from two independent experiments.

reported previously that both HCV and VSV enter host cells through low-pH-dependent endocytosis (7, 16, 20, 35, 42, 50). To determine whether HCVvsv entry was still dependent on endocytosis, we tested NH_4Cl and bafilomycin A1, inhibitors that prevent the acidification of endosomal compartments. HCVcc was used as a control for endocytosis-dependent entry, while HIV-based pseudoparticles bearing amphotropic murine leukemia virus (MLVpp) (54), which fuses directly at the plasma membrane at a neutral pH, was used as a control for endocytosis-independent entry. As shown in Fig. 5D, infection by HCVcc, HCVΔE, or HCVvsv was effectively blocked by pretreatment of cells with NH_4Cl or bafilomycin A1, while HIV-MLV infection was not affected. These data suggested that HCVvsv also requires a low-pH step for productive entry.

Buoyant densities of HCVΔE and HCVvsv. Next, we analyzed the buoyant densities of HCVcc, HCVΔE, and HCVvsv by sucrose density gradient analysis. After ultracentrifugation in a 20 to 60% sucrose gradient, the infectivity titers and HCV RNA contents of each density fraction were determined. As shown in Fig. 6, the infectivities of HCVΔE and HCVcc were distributed over a broad range of density fractions (90% of infectivity was recovered from 7 fractions between 1.02 and 1.14 g/ml), with a mean density of 1.08 g/ml. Notably, HCVΔE possessed more infectivity and genomic-RNA-containing viral particles in the low-density fractions (1.01 to 1.05 g/ml) than HCVcc, perhaps due to the reduced amounts of glycoproteins in the HCVΔE envelope. In contrast, the infectivity of HCVvsv was distributed over a smaller range of density fractions (90% of infectivity was recovered from 5 fractions between 1.08 and 1.15 g/ml), with a mean density of 1.10 g/ml. These results suggested that HCVvsv particles had a more homogeneous and

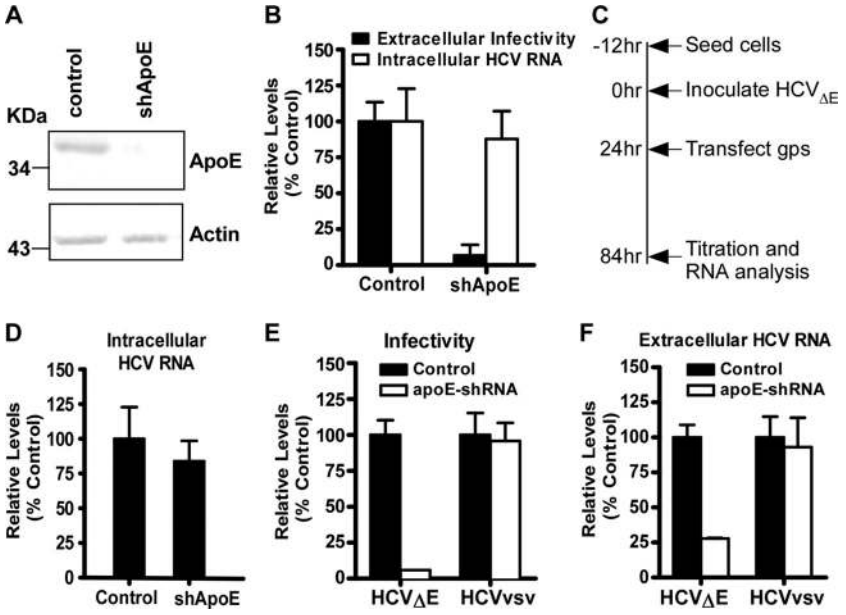

FIG. 7. Analysis of the dependency of HCVcc, HCVΔE, and HCVvsv secretion on apoE. (A) Western blot analysis of apoE expression in apoE knockdown (shApoE) and control cells. (B) apoE knockdown and control cells were infected with HCVcc at an MOI of 5. Extracellular infectivity and intracellular HCV RNA levels were determined by the titration assay and quantitative RT-PCR. (C) Protocol for the HCV secretion assay. apoE knockdown and control cells were infected with HCVΔE at an MOI of 0.5. At 24 h postinoculation, one portion of infected cells was collected for the determination of intracellular HCV RNA levels, and another portion of cells was transfected with a plasmid expressing JFH1 or VSV glycoproteins (gps). (D) Determination of intracellular RNA levels in order to compare HCVΔE infection efficiencies in the two cell lines. (E and F) The extracellular infectivity (E) and HCV RNA levels (F) of the supernatants from transfected cells were determined at 60 h posttransfection. The data are presented as percentages of the control level from two independent experiments performed in duplicate. Means ± standard deviations are shown.

heavier buoyant density profile than HCVΔE and HCVcc, possibly due to less association of HCVvsv particles with host lipoproteins.

apoE was not required for the secretion of infectious HCVvsv. It has been demonstrated recently that HCVcc secretion is associated with the host lipoprotein secretory pathway and that apoE, an important component of LDL/VLDL, is required for HCV production and infection (5, 9, 10, 19, 21, 23, 39). To assess the role of apoE in HCVΔE and HCVvsv production, we established a Huh7.5.1 apoE knockdown cell line, in which apoE expression was stably downregulated with an apoE-specific shRNA, and a control cell line that expressed an unrelated shRNA. The apoE shRNA led to a profound and stable reduction in apoE expression (Fig. 7A). Then we verified the effect of apoE knockdown on HCVcc production. The apoE knockdown and control cells were infected with HCVcc at an MOI of 5 as described in Materials and Methods. At 24 h postinfection, the cells were assayed for intracellular HCV RNA levels, and the culture supernatants were assayed for infectivity titers. As shown in Fig. 7B, the HCV RNA levels in the control and apoE knockdown cells were comparable, while extracellular infectivity titers were significantly reduced in apoE knockdown cells. This result was consistent with previous findings (5, 19, 23), clearly demonstrating that apoE is required for HCVcc secretion.

Next, we investigated whether HCVΔE and HCVvsv secretion also required apoE. As shown in Fig. 7C, apoE knockdown and control cells were inoculated with HCVΔE at an MOI of 0.5. At 24 h postinoculation, one portion of infected cells was collected for the determination of intracellular HCV RNA levels, in order to compare the HCVΔE infection efficiencies in the two cell lines, and another portion of cells was washed extensively with medium to remove the initial inocula and was then transfected with plasmids expressing the JFH1 or VSV glycoproteins. Extracellular infectivity and HCV RNA levels in the supernatants from the transfected cells were determined at 60 h posttransfection. Our results showed that the intracellular HCV RNA levels for the two cell lines were comparable (Fig. 7D). However, as shown in Fig. 7E and F, the knockdown of apoE expression significantly reduced the infectivity titers and total HCV RNA levels of HCVΔE in the supernatants but did not affect HCVvsv secretion at all. Collectively, these data demonstrated that apoE was required for HCVΔE secretion but not for HCVvsv secretion, suggesting that HCVvsv egress may not be associated with the host LDL/VLDL secretory pathway.

Establishment of HCV replicon cells by HCVvsv transduction. Our data showed that HCVvsv infection was not restricted by the normal HCV infection tropism, raising the possibility that HCVvsv could be used to deliver HCV RNA containing an antibiotic-selectable marker into cells that are nonpermissive for HCV entry. To test this possibility, we first transfected the envelope gene-deleted JFH1 replicon RNA containing a neomycin marker (Fig. 8A) into Huh7 cells in order to establish replicon cells that stably replicated the HCV genome. The established replicon cells were then transfected with a plasmid expressing VSV-G. Three days later, the culture supernatants were transferred to CD81-negative R3 cells in

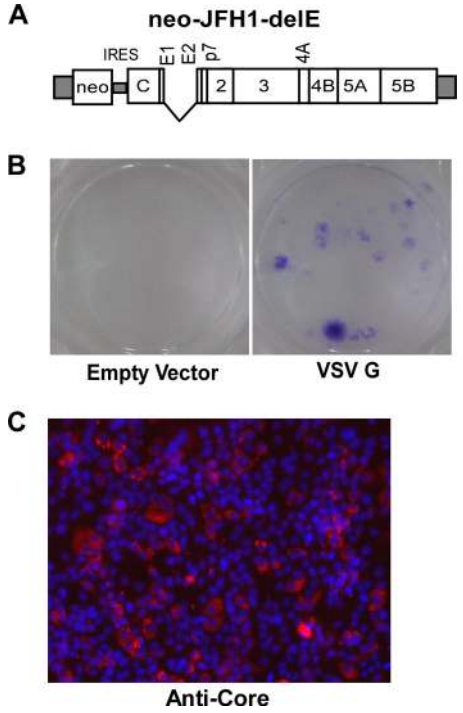

FIG. 8. Establishment of HCV replicon cells by HCVvsv transduction. (A) Structure of the JFH1 replicon RNA with the envelope genes deleted and containing a neomycin-selectable marker. (B) R3 cells ($CD81^-$ Huh7 derivative) were inoculated with culture supernatants of empty-vector- or VSV-G-transfected neo-JFH1-delE replicon cells. Inoculated cells were cultured for 4 weeks in a medium supplemented with G418 (800 μg/ml), and G418-resistant colonies were stained with crystal violet. The results shown are representative of two independent experiments. (C) Immunofluorescence analysis of HCV core (red) in the established replicon cell clone after HCVvsv transduction. Nuclei (blue) were stained with Hoechst dye.

order to select G418-resistant colonies. As shown in Fig. 8B, the culture supernatants from VSV-G-transfected cells produced visible colonies after 4 weeks of G418 selection, while the culture supernatants of cells transfected with the empty vector did not. The presence of HCV replicon RNA in the G418-resistant cells was further confirmed by immunostaining for HCV core (Fig. 8C) and by RT-PCR (data not shown).

DISCUSSION

In this study, we developed a new way to produce infectious HCV particles that encapsidate the HCV genome lacking the envelope-encoding regions (HCVΔE). Steinmann and coworkers showed previously that the luciferase-tagged JFH1 genome lacking the envelope-encoding regions alone could be rescued by a helper virus expressing the entire JFH1 genome, but virus production was low as determined by the luciferase assay, and it was not certain whether this virus lacking the envelope-encoding regions alone was able to expand and propagate in packaging cells, although these investigators did show that an HCV subgenomic replicon could be *trans*-complemented and that the viruses produced could be passaged in the packaging cell line that expressed all HCV structural proteins (47). Moreover, Pacini and coworkers showed that the envelope gene-deleted J6/JFH1 RNA could be rescued by the full-length J6/JFH1 genome or by the regions encoding the entire structural proteins (core-NS2) in *trans*, but not by the expression of envelope proteins alone in *trans* (40). During the revision of this article, Bianchi and colleagues also showed that the a E1E2-deficient JFH1 genome could be rescued by a packaging cell line expressing autologous envelope proteins (6). Our work was in agreement with their results and provided a more detailed characterization of these virus particles.

In addition, we found that the envelope proteins of J6CF (genotype 2a) could rescue JFH1-based HCVΔE production but that the envelope proteins of H77 (genotype 1a) and Con1 (genotype 1b) could not (Fig. 4), possibly due to intergenotypic incompatibility. Yi and colleagues reported that the adaptive mutation Q221L in the helicase domain of NS3 could rescue infectious virus production by compensating for the NS2-mediated assembly defect in the H77/JFH1 chimera (32, 55). However, our results showed that the production of HCVΔE with the Q221L mutation still could not be rescued by H77 envelope proteins (data not shown), suggesting that more molecular interactions between structural and nonstructural viral proteins may contribute to this intergenotypic incompatibility. Steinmann and coworkers also reported that a JFH1 subgenomic replicon (NS3-NS5B) could be *trans*-complemented by a helper virus expressing Con1 structural proteins, albeit with low efficiency (47). This result is contrast to our observation and could be explained by the fact that they provided the entire structural proteins (core-NS2) in *trans* from the same subtype, whereas we provided only the E1 and E2 proteins in *trans*. Thus, a more thorough understanding of the network of viral protein interactions involved in HCV assembly and secretion is needed in order to produce HCVΔE containing envelope proteins of a wide range of HCV genotypes.

The most interesting finding of our study is that foreign envelope glycoproteins (VSV-G) could substitute for HCV envelope function in viral assembly and entry. To our knowledge, this is the first study to demonstrate that VSV-G could form an envelope for HCV particles. This was an unexpected finding, given that HCV envelope proteins have been shown to be retained mainly on the ER membrane and that nascent HCV virions bud into the ER lumen, while VSV-G are localized mainly on the plasma membrane (20, 29, 52). Our further characterization showed that HCVvsv entry was indeed mediated by VSV-G and did not depend on HCV receptors. Interestingly, unlike that of HCVcc and HCVΔE, the release of HCVvsv particles was not affected by the knockdown of apoE, a critical host factor involved in HCV egress (5, 9, 19, 23). This may imply that HCVvsv exits cells independently of the host LDL/VLDL secretory pathway. In agreement with this finding, HCVvsv particles exhibit a narrower and heavier buoyant density distribution than HCVcc and HCVΔE. It has been shown that VSV-G could package a Semliki Forest virus (SFV) RNA replicon to produce virus-like particles, and this process involved the release of intracellular vesicles containing VSV-G and SFV RNA (13, 44, 46). It is possible that the release of HCVvsv particles may occur through a similar mechanism. Further studies will be required to determine the molecular mechanisms of HCVvsv morphogenesis.

HCVvsv can be used to deliver an HCV genome containing an antibiotic-selectable marker in order to establish replicon

cell lines. As a proof of concept, we showed that VSV-G easily delivered neo-JFH1-delE RNA into CD81-negative Huh7 cells and successfully established a neomycin-resistant HCV replicon cell line. Our approach has obvious advantages over the traditional electroporation-based method for establishing HCV replicon cell lines, since electroporation needs many cells and *in vitro*-synthesized HCV replicon RNA transcripts and is generally toxic to the host cells. Another advantage of the HCVvsv transduction-based approach is that VSV-G confers a wide host cell tropism. This is particularly useful in establishing HCV replicons in nonhepatic cell lines, since it has been shown that HCV could replicate in HeLa, HEK293, and mouse fibroblast cell lines (26, 51).

There is a great need for effective vaccines to prevent HCV infection. HCVΔE producing single-cycle infection in naïve cells should provide a new means of HCV vaccine development. The greatest advantage of HCVΔE is that this virus possesses all the structural viral proteins to induce humoral immune responses and that, upon infection, it could produce all the nonstructural viral proteins in host cells to induce cell-mediated immune responses. Of course, further studies, especially in animals, are needed to determine the immunogenicity, safety, and efficacy of an HCVΔE-based vaccine against HCV infection.

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